Amendments to the Specification:

Please replace the Title of the Invention with the following amended Title:

Improved Method for Measuring ~~Membrane~~ Transmembrane Potential Changes in a Biological Cell

Please replace the paragraph beginning at page 2, line 22 with the following amended paragraph:

The plasma membrane of a cell typically has a transmembrane potential of approximately -70 mV (negative inside) as a consequence of K^+, Na^+ and Cl^- concentration gradients that are maintained by active transport processes. Potentiometric probes offer an indirect method of detecting the translocation of these ions. Increases and decreases in membrane potential (referred to as membrane hyperpolarization and depolarization, respectively) play a central role in many physiological processes, including nerve-impulse propagation, muscle contraction, cell signaling and ion-channel gating (references 1-3). Potentiometric probes are important tools for studying these processes, and for cell-viability assessment. Potentiometric probes include the cationic or zwitterionic styryl dyes, the cationic carbocyanines and

rhodamines, the anionic oxonols and hybrid oxonols and merocyanine 540 (references 4-8). The class of dye determines factors such as accumulation in cells, response mechanism and toxicity. ~~Mechanism~~ Mechanisms for optical sensing of membrane potential have traditionally been divided into two classes: sensitive but slow redistribution of permanent ions from extracellular medium into the cell, and fast but small perturbation of relatively ~~impeable~~ impermeable dyes attached to one face of the plasma membrane (references 2 and 3).

Please replace the paragraph beginning at page 3, line 2 with the following amended paragraph:

The bis-barbituric acid and ~~thiobarturic~~ thiobarbituric oxonols, often referred to as DiBAC and DiSBAC dyes respectively, form a family of spectrally distinct potentiometric probes with excitation maxima covering most of the range of visible wavelengths. $DiBAC_4(3)$ and $DiSBAC_2(3)$ have been the two most popular oxonol dyes for membrane potential measurement (references 9 and 11). These dyes enter depolarized cells where they bind to intracellular proteins or membranes and exhibit enhanced fluorescence and red spectral shifts. Increased depolarization results in more influx of the anionic dye and thus an increase in fluorescence. $DiBAC_4(3)$ reportedly has the

highest voltage sensitivity. The long-wavelength $DiSBAC_2(3)$ has frequently been used in combination with the UV light-excitable Ca^{2+} indicators Indo-1 or Fura-2 for simultaneous measurements of membrane potential and Ca^{2+} concentrations. Interactions between anionic oxonols and the cationic K^+-valinomycin complex complicate the use of this ionophore to calibrate potentiometric responses. DIBAC and DiSBAC dyes are excluded from mitochondria because of their overall negative charge, making them superior to carbocyanines for measuring plasma membrane potentials.

Please replace the paragraph beginning at page 3, line 17 with the following amended paragraph:

In general, DiBAC and DiSBAC dyes bearing longer alkyl chains had been proposed to have better ~~propertie~~ properties for measuring membrane potentials (references 5 and 12). $DiSBAC_6(3)$ has been selected to use in a FRET-based membrane potential assay (reference 12). There are no reports on $DiBAC_1$ and $DiSBAC_1$ for measuring membrane ~~potentials~~ potential.

Please replace the paragraph beginning at page 3, line 22 with the following amended paragraph:

It has been discovered that ~~$DIBAC_1(3)$~~ $DiBAC_1(3)$ and $DiSBAC_1(3)$ ~~that~~ possess unexpected properties that can be used to measure membrane potentials with FLIPR and other fluorescence devices. Compared with other members of the DiBAC and DiSBAC family, ~~$DIBAC_1(3)$~~ $DiBAC_1(3)$ and $DiSBAC_1(3)$ give stronger signal and faster response besides its better water solubility.

Please replace the paragraph beginning at page 4, line 4 with the following amended paragraph:

The invention encompasses an improved method for measuring membrane potential using compounds of the formula I as potentiometric probes. These probes may be used in combination with other fluorescent indicators such as Indo-1, Fura-2, and Fluo-3, calcium green or Fluo-4. Such probes may be used in microplate reading devices such as FLIPR, fluorescent imaging plate ~~reader,sold~~ reader, sold by Molecular ~~Devicе~~ Devices Corp., of Sunnyvale, Calif.; flow cytometers; and fluorometers. Such probes are used to measure membrane potential in live cells.

Please replace the paragraph beginning at page 4, line 22 with the following amended paragraph:

The invention also encompasses test ~~kit~~ kits containing reagents of compound I, compound I in combination with ~~a~~ another fluorescent reagent and in particular fluorescent indicators such as Indo-1, Fura-2, Fluo-3, calcium green or Fluo-4.

Please replace the paragraph beginning at page 4, line 29 with the following amended paragraph:

(a) A first reagent selected from the potentiometric probes which redistribute from one side of the membrane to the opposite side in response to transmembrane potential; and a second reagent selected from the group consisting of non-fluorescent dyes or pigments that ~~re~~ are not membrane-permeable, and undergo energy transfer with the first reagent on one side of the ~~membranes~~ membrane to reduce or eliminate the fluorescence signal on that side; or

Please replace the paragraph beginning at page 5, line 4 with the following amended paragraph:

(b) A first reagent selected from the potentiometric probes which redistribute from one side of the membrane to the opposite side in response to transmembrane potential; and a second reagent selected from the group consisting of non-fluorescent dyes or pigments that ~~re~~ are not membrane-permeable, and absorb the excitation light or emission from the first reagent on one side of the ~~membranes~~ membrane to reduce or eliminate the undesired fluorescence signal; or

Please replace the paragraph beginning at page 6, line 7 with the following amended paragraph:

FIG. 1 is a reaction scheme for making DiBAC and ~~DISBAC~~ DiSBAC.

Please replace the paragraph beginning at page 6, line 12 with the following amended paragraph:

FIG. 3 illustrates the absorption spectra of ~~DisBAC$_1$(3)~~ DiSBAC$_1$(3), ~~DIsBAC$_2$(3)~~ DiSBAC$_2$(3), ~~DisBAC$_4$(c)~~ DiSBAC$_4$(3), ~~DIBAC$_1$(3)~~ DiBAC$_1$(3) and DiBAC$_4$(3) in 1:1 methanol/water.

Please replace the paragraph beginning at page 6, line 15 with the following amended paragraph:

FIG. 4 illustrates the fluorescence spectra of ~~$DisBAC_1(3)$~~ $DiSBAC_1(3)$, ~~$DisBAC_2(3)$~~ $DiSBAC_2(3)$, ~~$DisBAC_3(3)$~~ $DiSBAC_3(3)$, $DiBAC_4(3)$, ~~$DIBAC_4(3)$~~ $DiBAC_1(3)$ and $DiBAC_4(3)$ in 1:1 methanol/water.

Please replace the paragraph beginning at page 7, line 16 with the following amended paragraph:

DiBAC and DiSBAC dyes are prepared based on the procedure for ~~of~~ ethyl and butyl derivatives[[)]] (H. Bartsch and G. Haubold, Arch. Pharm. 1982, 315, 761-766). Specifically, malonaldehyde bis(phenylimine)monohydrochloride (2.6 g, 10 mmol) and 1,3-dimethyl-2-thiobarbituric acid (3.5 g, 20 mmol) are dissolved in acetonitrile (40 mL). To the solution is added triethylamine (2 g, 20 mmol). The reaction mixture is refluxed until the starting materials are completely consumed as indicated by TLC. The mixture is cooled to room temperature, and poured into acidic water (pH 2-3, 350 mL). The resulting suspension is filtered to collect the solid that is washed with cold water and air-dried. The crude product is further purified on a silica gel column

using a gradient of ~~dichoromethano/metahnol~~ dichloromethanol/methanol to give the desired product.

Please replace the paragraph beginning at page 9, line 18 with the following amended paragraph:

~~DisBAC$_1$(3)~~ DiSBAC$_1$(3), ~~DisBAC$_2$(3)~~ DiSBAC$_2$(3), ~~DisBAC$_3$(3)~~ DiSBAC$_3$(3), ~~DisBAC$_4$(3)~~ DiSBAC$_4$(3), DiBAC$_1$(3) and DiBAC$_4$(3) are dissolved in DMSO (3 mM). The DMSO stock solutions are respectively ~~partrioned~~ partitioned in a 1:1 octanol/water mixture. The concentrations of the oxonol dyes in octanol and water layers are determined by absorption spectra. The results are summarized in the following table I. As shown in the table, ~~DisBAC$_1$(3)~~ DiBAC$_1$(3) and ~~DisBAC$_1$(3)~~ DiSBAC$_1$(3) are much more hydrophilic than the other oxonol dyes. They also have much better water solubility.

Please replace the paragraph beginning at page 9, line 29 with the following amended paragraph:

~~DisBAC$_1$(3)~~ DiSBAC$_1$(3), ~~DisBAC$_2$(3)~~ DiSBAC$_2$(3), ~~DisBAC$_3$(3)~~ DiSBAC$_3$(3), ~~DisBAC$_4$(3)~~ DiSBAC$_4$(3), DiBAC$_1$(3) and DiBAC$_4$(3) are dissolved in methanol (1 mM). The stock solutions are diluted with 1:1 methanol/water, and the absorption spectra are recorded

in a spectrophotometer. As shown in FIG. 3, $DiBAC_1(3)$ and ~~$DisBAC_1(3)$~~ $DiSBAC_1(3)$ possess unexpected blue shift compared to the other oxonol dyes.

Please replace the paragraph beginning at page 10, line 1 with the following amended paragraph:

~~$DisBAC_1(3)$~~ $DiSBAC_1(3)$, ~~$DisBAC_2(3)$~~ $DiSBAC_2(3)$, ~~$DisBAC_3(3)$~~ $DiSBAC_3(3)$, ~~$DisBAC_4(3)$~~ $DiSBAC_4(3)$, $DiBAC_1(3)$ and $DiBAC_4(3)$ are dissolved in methanol (1 mM). The stock solutions are diluted with 1:1 methanol/water, and the absorption spectra are recorded in a fluorometer. As shown in FIG. 4, $DiBAC_1(3)$ and ~~$DisBAC_1(3)$~~ $DiSBAC_1(3)$ possess unexpected blue shift compared to the other oxonol dyes.

Please replace the paragraph beginning at page 10, line 8 with the following amended paragraph:

~~$DisBAC_1(3)$~~ $DiSBAC_1(3)$, ~~$DisBAC_2(3)$~~ $DiSBAC_2(3)$, ~~$DisBAC_3(3)$~~ $DiSBAC_3(3)$, ~~$DisBAC_4(3)$~~ $DiSBAC_4(3)$, $DiBAC_1(3)$ and $DiBAC_4(3)$ are dissolved in DMSO (1 mM). The stock solutions are respectively used to assay membrane potential changes in the P2X2 cells in combination with FLIPR™ as described in Example 2. The results are summarized in the following table 2.

Please replace Table 2, which begins at page 10, line 13 with the following amended Table 2:

Compounds	Fluorescence Enhancement by 10 μM ATP stimulation (in folds)	Response speed
$DiBAC_1(3)$	3.7	fast
$DiBAC_4(3)$	3.3	slow
~~$DisBAC_1(3)$~~ $DiSBAC_1(3)$	108.5	fast
~~$DisBAC_2(3)$~~ $DiSBAC_2(3)$	38.6	moderate
~~$DisBAC_3(3)$~~ $DiSBAC_3(3)$	14.5	slow
~~$DisBAC_4(3)$~~ $DiSBAC_4(3)$	2.3	slow

Table 2

Please replace the paragraph beginning at page 10, line 16 with the following amended paragraph:

As shown in the table 2, ~~$DisBAC_1(3)$~~ $DiSBAC_1(3)$ is much more sensitive, and has faster response to membrane potential change than the rest of the ~~DisBACs~~ DiSBACs. $DIBAC_1(3)$ also has much faster response to membrane potential change than $DiBAC_4(3)$.

Please replace the paragraph beginning at page 10, line 22 with the following amended paragraph:

Protocols for transmembrane potential measurements are summarized briefly since they are similar to those given in

detail in Example I above. The Bis-(1,3-dimethylthiobarbituric acid) trimethine oxonol, [$DiSBAC_1(3)$], fluorescent reagent may be purchased from Molecular Probes (Eugene, ~~Oreg.~~ OR, USA). The 1.times. Cell-Loading Buffer for $DiSBAC_2(3)$, consists of sodium-free Tyrode's Buffer (SFTB), 2.5 uM $DiSBAC_1(3)$, and 200 uM Direct Blue 71 (as the fluorescence quencher).

Please replace the paragraph beginning at page 10, line 29 with the following amended paragraph:

A rat pheochromocytoma (adrenal) cloned cell line, PC12, is grown in RPMI 1640 culture medium with 10% fetal calf serum (FCS), 1% penicillin/streptomycin (PS), 2 mM L-glutamine, and 1 mM sodium pyruvate. Cells were grown in suspension, and subsequently centrifuged from growth medium and resuspended in $DiSBAC_2(3)$+, 1X Cell-Loading Buffer. Approximately 100,000 cells were plated per well in a 96 well microtiter plate pre-coated with poly-D-lysine to ~~enchance~~ enhance cell adhesion, centrifuged at 1000 rpm for 4 minutes, and placed in an incubator for an additional 20 minutes. Cells were not washed with any liquid medium, nor was the 1X Cell-Loading Buffer removed prior to performing fluorescence measurements.

Please replace the paragraph beginning at page 11, line 18 with the following amended paragraph:

The above examples illustrate the present invention and are not intended to limit the invention in spirit ~~of~~ or scope.